PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :		(11) International Publication Number:
A61K 39/395, G01N 33/577 C12N 5/00, A61K 49/00	A1	WO 89/ 05155
		(43) International Publication Date: 15 June 1989 (15.06.89)

(21) International Application Number: PCT/US88/04118

(22) International Filing Date: 17 November 1988 (17.11.88)

(31) Priority Application Number: 123,045

(32) Priority Date: 19 November 1987 (19.11.87)

(33) Priority Country: US

(71) Applicant: SCRIPPS CLINIC AND RESEARCH FOUNDATION [US/US]; 10666 North Torrey Pines Road, La Jolla, CA 92037 (US).

(72) Inventor: CHERESH, David, A. ; 5076 Park West Avenue, San Diego, CA 92117 (US).

(74) Agents: GAMSON, Edward, P.; Dressler, Goldsmith, Shore, Sutker & Milnamow, Ltd., 1800 Prudential Plaza, Chicago, IL 60601 (US) et al.

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: MONOCLONAL ANTIBODY AGAINST THE RGD-DIRECTED ADHESION RECEPTOR OF ENDOTHELIAL CELLS

Amino Acid Sequence of the Amino Terminus
of RGD-Directed Adhesion Receptors

```
                    5         10        15        20

ALPHA SUBUNIT

                      D G                 V
ECr     F N L D V E S P A E Y S G P E G S Y F
VNR     F N L D V X S P A E Y S
GpIIb   L N L D P V Q L T F Y A G P N G S Q F G F S

BETA SUBUNIT

                    S                   G
ECr     G P N I C T T R G V S S C Q Q C L
VNR     G P N I C T T S G V S S C Q
GpIIIa  G P N I C T T R G V S S C Q
```

(57) Abstract

A monoclonal antibody that immunoreacts with ECr, the RGD-directed adhesion receptor found on the surface of both endothelial and melanoma cells, is disclosed. Methods whereby that monoclonal antibody can be used to inhibit the ability of cells that contain the adhesion receptor to adhere to a subendothelial matrix composed of vitronectin, fibrinogen or von Willebrand factor are also disclosed. Further disclosed are diagnostic systems and methods for assaying for ECr in a body sample using the antibody of the invention.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**ML**	Mali
AU	Australia	**GA**	Gabon	**MR**	Mauritania
BB	Barbados	**GB**	United Kingdom	**MW**	Malawi
BE	Belgium	**HU**	Hungary	**NL**	Netherlands
BG	Bulgaria	**IT**	Italy	**NO**	Norway
BJ	Benin	**JP**	Japan	**RO**	Romania
BR	Brazil	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SN**	Senegal
CH	Switzerland	**LI**	Liechtenstein	**SU**	Soviet Union
CM	Cameroon	**LK**	Sri Lanka	**TD**	Chad
DE	Germany, Federal Republic of	**LU**	Luxembourg	**TG**	Togo
DK	Denmark	**MC**	Monaco	**US**	United States of America
FI	Finland	**MG**	Madagascar		

A MONOCLONAL ANTIBODY AGAINST THE RGD-DIRECTED ADHESION RECEPTOR OF ENDOTHELIAL CELLS

Description

Technical Field

The present invention relates to a monoclonal antibody that immunoreacts with the RGD-directed adhesion receptor of endothelial cells (ECr), and thereby inhibits the binding of endothelial cells to the RGD-containing matrix proteins vitronectin, fibrinogen and von Willebrand factor. Also contemplated are therapeutic methods wherein a monoclonal antibody of the present invention is used to inhibit the adhesion of endothelial cells to the subendothelial matrix and thereby modulate tumor growth.

Background

Cell adhesion is a critical process in tumor growth because it plays a role in the formation and vascularization of new tumor tissue. Therefore, agents that inhibit cell adhesion can be used therapeutically to inhibit tumor growth.

Cell adhesion generally involves recognition of specific adhesive proteins by cell surface receptors. The cell surface receptors of present interest specifically bind to matrix proteins at sites that have as a part of their structure the tripeptide amino acid residue sequence Arg-Gly-Asp (RGD). Such receptors are known in the art as RGD-directed adhesion receptors. The binding specificity of individual RGD-directed adhesion receptors appears to depend on the structural milieu within which the RGD tripeptide is found. RGD-directed adhesion receptors are further characterized as consisting of non-covalently associated alpha and beta subunits, i.e.,

they are heterodimeric protein complexes. Exemplary RGD-directed adhesion receptors are vitronectin receptor, fibronectin receptor and GpIIb-IIIa, all of which are distinguishable on the basis of their subunit composition and the proteins which they specifically bind.

Of particular interest to the present invention is the RGD-directed adhesion receptor, designated ECr, first found on M21 human melanoma cells by Cheresh et al., J. Biol. Chem., 262:1434-37 (1987). According to Cheresh et al., ECr isolated from M21 cells is a heterodimer consisting of alpha and beta subunits which under reducing conditions display molecular weights of about 130 kilodaltons (kDa) and 105 kDa, respectively, when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Two monoclonal antibodies capable of immunoreacting with ECr have been produced and described by Dr. David Cheresh, the present inventor, at the 26th Annual Meeting of the American Society for Cell Biology in December, 1986. One of those monoclonal antibodies, designated LM609, was reported by Cheresh to be capable of blocking the ability of M21 cells to bind fibrinogen, vitronectin and von Willebrand factor. Thus, ECr appears to mediate adhesion of M21 cells to fibrinogen, vitronectin and von Willebrand factor, all of which are substrate associated proteins that contain RGD as part of their amino acid residue sequence.

More recently, it has been reported that ECr is also found on the surface of human umbilical vein endothelial (HUVE) cells. The evidence in support of that finding is at least two-fold and was reported by Cheresh at the Gordon Conference Meeting on

Fibronectin and Related Proteins in February 1987. First, monoclonal antibody LM609 immunoreacts with a heterodimeric RGD-directed receptor on HUVE cells that has alpha and beta subunits displaying molecular weights indistinguishable from ECr isolated from M21 cells. Second, monoclonal antibody LM609 immunoreacts with HUVE cells and thereby inhibits their binding to fibrinogen, vitronectin and von Willebrand factor, indicating that the RGD-directed receptor recognized by LM609 on HUVE cells has the same ligand specificity (matrix protein binding profile) as ECr.

The ability to block the adhesion of HUVE cells to fibrinogen, vitronectin and von Willebrand factor using a monoclonal antibody against a RGD-directed adhesion receptor has also been reported by Charo et al., J. Biol. Chem., 262:9935-38 (1987). According to Charo et al., a monoclonal antibody, designated 7E3, raised against GpIIb-IIIa, an RGD-directed adhesion receptor found on human platelets, could immunoreact not only with GpIIb-IIIa, but also with a heterodimeric RGD-directed adhesion receptor on HUVE cells having alpha and beta subunits displaying molecular weights of about 130 kDa and 105 kDa, respectively, i.e., molecular weights similar to those of ECr but distinguishably different from GpIIb-IIIa. Thus, ECr and GpIIb-IIIa appear to share at least one immunologically cross-reactive epitope.

Monoclonal antibody 7E3 was also shown by Charo et al. to be capable of immunoreacting with HUVE cells and blocking their attachment to substrates containing fibrinogen and von Willebrand factor, and partially blocking their attachment and spreading on vitronectin, thus indicating that the receptor recognized by 7E3 on HUVE cells has a similar matrix protein binding profile as ECr.

Brief Summary of the Invention

The present invention provides a monoclonal antibody comprising antibody molecules that immunoreact with ECr present on HUVE cells and thereby inhibits the adhesion of those cells to vitronectin, fibrinogen or von Willebrand factor. Preferably, the anti-ECr antibody present in the monoclonal antibody are those produced by the hybridoma having ATCC designation HB9537.

Thus, the present invention contemplates a hybridoma that produces antibody molecules that immunoreact with ECr present on HUVE cells and thereby inhibits the adhesion of those cells to vitronectin, fibrinogen or von Willebrand factor.

Also contemplated is a method of inhibiting tumor growth in a subject. The method comprises administering, in a pharmaceutically acceptable excipient a therapeutically effective amount of a monoclonal antibody comprising antibody molecules that immunoreact with ECr present on HUVE cells and thereby inhibits the binding of those cells to vitronectin, fibrinogen, and von Willebrand factor.

Further contemplated by the present invention is a method of assaying for the presence of ECr in a body sample. The method comprises forming an immunoreaction admixture by admixing a body sample to be assayed with a monoclonal antibody of the present invention. The admixture is maintained for a time period sufficient for any ECr present in the sample to immunoreact with the monoclonal antibody to form a ECr-antibody immunoreaction product. Assaying for the presence of any ECr-antibody immunoreaction product formed thereby provides a means for detecting the presence ECr in the sample.

Still further contemplated is a method of assaying in vivo for the presence of ECr in a subject. The method comprises intravenously administering to a subject a diagnostically effective amount of a monoclonal antibody of the present invention that is linked to an in vivo indicating means and dispersed in a physiologically tolerable excipient. The subject is then maintained for a predetermined time period sufficient for the administered monoclonal antibody to immunoreact in vivo with any ECr present in the subject to form an immunoreaction product. Assaying for the presence and preferably the location of any immunoreaction product formed within the subject thereby provides a means for detecting the presence of ECr in vivo.

The present invention also contemplates a diagnostic system in kit form useful for performing the contemplated assay methods. The system comprises a container that includes a monoclonal antibody of the present invention present in an amount sufficient to carry out at least one assay.

Thus, the present invention provides several benefits and advantages. One benefit provided by the present invention is that the contemplated monoclonal antibody provides a means for detecting the presence of ECr protein in a body sample and in a subject in vivo. In addition, because the monoclonal antibody of the present invention inhibits adhesion of ECr-containing cells to vitronectin, fibrinogen and von Willebrand factor, the present invention provides a therapeutic composition for inhibiting tumor cell growth.

An advantage provided by the present invention derives from the specificity that the contemplated monoclonal antibody has for ECr and not

VNr or FNr. Thus, contemplated methods for detecting the presence of ECr in body samples and in vivo can advantageously distinguish ECr from other RGD-directed adhesion receptors such as VNr or FNr. Further, the immunoreaction specificity of the contemplated monoclonal antibody limits the effects of inhibition of cell adhesion to only those cells bearing ECr.

Brief Description of the Drawings

Figure 1 illustrates an autofluorogram of the immunoreaction product that forms with ECr present in M21 cell lysates using the ECr-specific monoclonal antibody that contains antibody molecules produced by hybridomas LM142 or LM609. The M21 cell proteins, including ECr, had ^{35}S-methionine labeled by pulsing a M21 cell culture with ^{35}S-methionine for 10 min and then chasing with unlabeled L-methionine for either 0 hours, i.e., zero time cells (lanes A and C), or for 8 hours (lanes B and D). The resulting immunoreaction products were electrophoresed under reducing conditions in a 7.5% polyacrylamide gel as described in Example 4.

As shown in lane A, Mab LM142 immunoreacts with a protein of about 160 kDa in molecular weight previously shown to be a biosynthetic precursor to the M21 cell ECr alpha subunit. Cheresh et al., J. Biol. Chem., 262:1434-37 (1987). After 8 hours of chase, Lane B illustrates that ECr immunoreacts with Mab LM142 and is comprised of alpha and beta subunits of 130 kDa and 105 kDa, respectively. Similar results, shown in lanes C and D, were obtained using Mab LM609.

Figure 2 contains six panels that graphically illustrate the effects of Mab LM609 on the kinetics of M21 cell attachment to microtiter wells each coated with one of five different matrix proteins or the peptide GRGPSPC (adhesion substrates).

3H-leucine labeled M21 cells were first immunoreacted with 0.05 mg/ml of either Mab LM609 (0) or heterologous Mab W6/32 (0). The antibody treated cells (Mab-cell immunoreaction products) were then plated into the coated microtiter wells and maintained for the indicated times to allow the cells to adhere to the wells as described in Example 5. The results are expressed as the total number of cells bound, that number being a function of the cell associated label, expressed in counts per minute (cpm X 10^{-3}) bound to the various matrix proteins after the indicated times. Each point represents the mean $\pm$ one standard deviation of five replicate measurements. The three lower panels demonstrate that Mab LM609 immunoreacts with and significantly inhibits the adhesion of M21 cells to, from left to right, von Willebrand factor, fibrinogen and the peptide GRGDSPC, as evidenced by the lower number of cpm detected for Mab LM609 as opposed to heterologous Mab W6/32.

Figure 3 is a composite figure comprising a graph in the lower portion and an autofluorogram in the upper portion that together illustrate the elution profile of HUVE cell ECr isolated on a GRGDSPK-Sepharose affinity column (purified ECr) as described in Example 6. An octylglucoside extract of HUVE cells whose proteins had been metabolically labeled with ^{35}S-methionine was passed over a GRGDSPK-Sepharose affinity matrix so as to allow any RGD-directed adhesion receptors present to bind to the matrix. The matrix was then washed first with elution buffer containing 1 mg/ml of the peptide GRGESP to yield eluted fractions 1 through 10, and then with elution buffer containing the peptide GRGDSP to yield fractions 11-20.

The graph illustrates the elution profile of ^{35}S-methionine labeled proteins determined by measuring the counts per minute (cpm) of radioactivity present in a 50 ul aliquot of each fraction using a liquid scintillation counter. The arrows mark the fraction in which the addition of elution buffer containing the indicated peptide to the affinity column was made.

The autofluorogram illustrates the elution profile of labeled extracts when analyzed by electrophoresis on 7.5% polyacrylamide gels under reducing conditions. The autofluorogram is aligned in the figure such that the lane of the gel is directly above the eluted fraction from which a 50 ul aliquot was taken for electrophoresis. The apparent molecular weights (Mr) of the two protein bands found in fractions 14-18, which represent the alpha (upper) and beta (lower) subunits, respectively, are indicated in kilodaltons.

Figure 4 illustrates an autofluorogram of a 7.5% polyacrylamide gel that was used to analyze the ability of Mab LM609 (lane A), Mab LM142 (lane B) and heterologous Mab 9.2.27 (lane C) to immunoreact with ECr from HUVE cells. As shown in lanes A and B, Mab LM609 and Mab 142 each immunoreacted with ECr to produce an immunoreaction product containing the ECr alpha and beta subunits having molecular weights of about 135 kDa and 115 kDa, respectively. The absence of detectable protein in lane C indicates that heterologous Mab 9.2.27 (negative control) did not immunoreact with ECr.

As shown in lanes A and B, Mab LM609 and Mab LM142 each immunoreact with the endothelial ECr heterodimer complex comprised of alpha and beta subunits of about 135 kDa and 115 kDa in molecular

weight, respectively. The heterologous antibody 9.2.27 does not immunoreact with purified ECr.

Figure 5 is a series of eight panels (A-H) that contain graphs illustrating the effects of Mab LM609 on the kinetics of HUVE cell attachment to various matrix proteins (adhesion substrates). ^{3}H-leucine labeled endothelial cells were first allowed to immunoreact with 0.05 mg/ml of Mab LM609 (0), heterologous Mab W6/32 (0) or growth medium alone ( ), washed free of excess antibody, and were then plated onto microtiter wells and maintained for the indicated times to permit adhesion to the matrix proteins that had been coated onto the microtiter wells as described in Example 8. The matrix proteins used were fibronectin (A), collagen IV (B), laminin (C), vitronectin (D), GRGDSPC (E), von Willebrand factor (vWF), pool I (F), vWF, pool III (G), and fibrinogen (H). The results are expressed in the same manner as described for Figure 2.

Panels D-H demonstrate that Mab LM609 immunoreacts with and significantly inhibits the adhesion of HUVE cells to the adhesion substrates vitronectin, von Willebrand factor, fibrinogen and the peptide GRGDSPC as evidenced by a reduction in the number of cells bound after treatment with Mab LM609 as opposed to Mab LM142 or heterologous Mab W6/32 (negative control).

Figure 6 illustrates the partial amino acid residue sequence of the amino terminal portion of M21 cell ECr protein, shown from left to right and in the direction from amino-terminus to carboxy-terminus using the single letter code beginning with residue 1 as the first residue on the amino terminus. The amino acid residue sequence of both the alpha and beta subunit of ECr was determined as described in

Example 9. Also shown are sequences of homologous regions of the vitronectin receptor alpha subunit [Ginsberg et al., J. Biol. Chem., 262:5437-40 (1987)], vitronectin receptor beta subunit [Takada et al., Proc. Natl. Acad. Sci. USA, 84:3239-43 (1987)], platelet GpIIb alpha subunit [Poncz et al., J. Biol. Chem., 262:8476-82 (1987)] and platelet GpIIIa beta subunit. Two different amino acid residues were found at residue positions 6, 7 and 16 of the alpha subunit and positions 5 and 16 of the beta subunit of ECr. The alternative residue is shown directly above the position at which it is found. This data indicates that ECr is similar but not identical to the vitronectin receptor.

Figure 7 is a bar graph illustrating the immunoreactivity of each of 5 Mabs with various tumor cell types as determined using the ELISA assay described in Example 10. The Mabs used include: LM142 and LM609, directed to ECr; AP3, directed to platelet GpIIIa beta subunit; LM543, directed to fibronectin receptor beta subunit; and, W6/32, directed to a human histocompatibility antigen. As shown in the panel beneath "LM609", Mab LM609 immunoreacts significantly (>0.1 $O.D._{492}$) with the following cell lines: M21, M16A, M18, M14, A375MM, Melur and WM266. Beneath "LM142", it is shown that Mab LM142 immunoreacts significantly with the following cell lines: M21, M16A, M18, M14, A375P, A375MM, Melur, WM266, WM239a, SK-N-AS, UCLA P3, T291, Bachle, SCL-I185, Colo 357 FG and U87 MG.

Figure 8 illustrates an autofluorogram of a 7.5% polyacrylamide gel that was used to analyze immunoreaction products produced in a study to determine whether or not the determinants recognized by Mab LM142 (panel A), Mab LM609 (panel B) and Mab

AP3 (panel C) were complex-dependent. The pulse-chase sample times are indicated in minutes (') above panels B and C. The sample times are the same for panel A as is indicated in the corresponding panel directly below in Panel B.

In panel A, Mab LM142 is shown to have formed an immunoreaction product that contains ECr alpha subunit precursor of about 160 kDa when immunoreacted with M21 cell pulse-chase lysates prepared at time intervals ranging from zero time point (0) to 240 minutes (240'). After a chase of approximately 120 minutes (120'), Mab LM142 produced immunoreaction products that contain 130 kDa and 105 kDa proteins corresponding to the alpha and beta subunits, respectively. Mab LM142 is directed to the alpha subunit of ECr and is characterized herein as having the ability to immunoreact with a complex-independent determinant.

In panel B, Mab LM609 is shown to have formed an immunoreaction product that contains the alpha subunit precursor when immunoreacted with the same M21 cell pulse-chase lysates as is shown in Panel A. The precursor first appears after a chase of about 5 minutes (5') beyond the zero time point and continues to be detected throughout the remaining chase period to 240 minutes. Mab LM609 also produced immunoreaction products after a 120 minute chase that contain the same 130 kDa and 105 kDa proteins detected when using Mab LM142 shown in panel A. Mab LM609 is directed to the heterodimer complex ECr and is characterized herein as having the ability to immunoreact with a complex-dependent determinant.

Mab AP3 is shown in panel C to have formed an immunoreaction product containing both the alpha subunit precursor and the 105 kDa beta subunit protein

when immunoreacted with the same M21 cell pulse-chase lysates as shown in Panel A. The precursor and the 105 kDa proteins each appear after a chase of about 10 minutes (10') and persist throughout the chase period to 120 minutes (120'). Mab AP3 is directed to the platelet glycoprotein IIIa but is also shown here to immunoreact with a determinant present on the beta subunit of ECr.

Detailed Description of the Invention

A. Definitions

Amino Acid: All amino acid residues identified herein are in the natural L-configuration. In keeping with standard polypeptide nomenclature, J. Biol. Chem., 243:3557-59, (1969), abbreviations for amino acid residues are as shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

SYMBOL		AMINO ACID
1-Letter	3-Letter	
Y	Tyr	L-tyrosine
G	Gly	glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
I	Ile	L-isoleucine
L	Leu	L-leucine
T	Thr	L-threonine
V	Val	L-valine
P	Pro	L-proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
W	Trp	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagine
C	Cys	L-cysteine
X	---	(unidentified residue)

It should be noted that all amino acid residue sequences are represented herein by formulae whose left to right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a bond to a further sequence of one or more

amino acid residues up to a total of about fifty residues in the polypeptide chain.

Polypeptide and Peptide: Polypeptide and peptide are terms used interchangeably herein to designate a linear series of no more than about 50 amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues.

Protein: Protein is a term used herein to designate a linear series of greater than 50 amino acid residues connected one to the other as in a polypeptide.

B. Monoclonal Antibody Compositions

The term "antibody" in its various grammatical forms is used herein as a collective noun that refers to a population of immunoglobulin molecules and/or immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antibody combining site or paratope.

An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope, including those portions known in the art as Fab, Fab', $F(ab')_2$ and F(v).

Fab and $F(ab')_2$ portions of antibodies are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibodies by methods that are well known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous and Dixon. Fab' antibody portions are also well known and are produced from $F(ab')_2$ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules are preferred, and are utilized as illustrative herein.

The phrase "monoclonal antibody" in its various grammatical forms refers to a population of one species of antibody molecule of determined (known) antigen-specificity. A monoclonal antibody contains only one species of antibody combining site capable of immunoreacting with a particular antigen and thus typically displays a single binding affinity for that antigen. A monoclonal antibody may therefore contain a bispecific antibody molecule having two antibody combining sites, each immunospecific for a different antigen.

A monoclonal antibody (Mab) of the present invention is characterized as containing, within detectable limits, only one species of antibody combining site capable of immunologically binding (immunoreacting with) the RGD-directed adhesion receptor referred to herein as ECr. Thus, the antibody molecules present in a monoclonal antibody of this invention typically display a single binding affinity for ECr even though it may contain antibody combining sites capable of binding proteins other than ECr.

Preferably, a monoclonal antibody of the present invention is further characterized as being substantially free of antibody molecules that immunoreact with vitronectin receptor (VNr) or fibronectin receptor (FNr) described below. That is, a monoclonal antibody of this invention does not, within the limits of immunological assay procedures, immunoreact with VNr or FNr, either in isolated form or present in the lysate of a cell that expresses either or both of those receptors. Both VNr and FNr are well known in the art and can be isolated by known methods from the human osteosarcoma cell line MG-63, available from The American Type Culture Collection, Rockville, MD under the designation ATCC CRL 1427. See for example Pytela et al., Cell, 40:191-8 (1985) and Pytela et al., Proc. Natl. Acad. Sci. USA, 82:5766-70 (1985).

More preferably, the anti-ECr antibody molecules present in a monoclonal antibody of the present invention are further characterized as having the ability to immunoreact with endothelial cells such as HUVE cells, bovine aortic endothelial (BAE) cells, chick embryo endothelial (CEE) cells and the like and thereby inhibit their adhesion (attachment) to vitronectin, fibrinogen, von Willebrand factor or a hexapeptide having the amino acid residue sequence GRGDSP. That is, a monoclonal antibody of this invention immunoreacts with ECr when it is present on the endothelial cell surface to form a cell-antibody complex that does not bind to vitronectin, fibrinogen, von Willebrand factor or the hexapeptide GRGDSP.

Preferably, the anti-ECr antibody molecules present in a monoclonal antibody of the present invention are still further characterized as having the ability to immunoreact with a complex-dependent

determinant present on ECr but incapable of immunoreaction with either the alpha or beta subunits when those subunits are not associated together in a ECr heterodimer complex.

An "antigenic determinant" or "determinant" is that structural portion of an antigen to which an antibody combining site immunologically binds. A determinant is "complex-dependent" when it is present on the ECr heterodimer complex comprised of non-covalently associated alpha and beta subunits but not present on either the alpha or beta subunits when the subunits have not associated into a heterodimer complex.

A preferred monoclonal antibody of this invention contains antibody molecules capable of being produced by the hybridoma LM609 (ATCC HB 9537), i.e., monoclonal antibody LM609 (Mab LM609). Thus, it is understood that while a preferred method for producing a monoclonal antibody of the present invention involves the use of hybridoma LM609, it is to be understood that a monoclonal antibody of the present invention can be produced by other well known methods. Those methods include the use of a hybridoma or other cell formed by cell-fusion that produces anti-ECr antibody molecules functionally equivalent to those produced by hybridoma LM609, preferably a hybridoma that produces an anti-ECr antibody that has a paratope that is immunologically cross-reactive with the anti-ECr paratope produced by hybridoma LM609. Those methods also include the use of any cell that contains an immunoglobulin heavy- or light-chain variable region-encoding DNA sequence that is homologous to the anti-ECr antibody heavy- or light-chain variable region-encoding DNA sequence present in hybridoma LM609. Thus, the present invention contemplates any

monoclonal antibody that is functionally the same as a monoclonal antibody of this invention produced through the use of hybridoma LM609.

A monoclonal antibody of the present invention can be produced by initiating a monoclonal hybridoma culture comprising a nutrient medium containing a hybridoma of the present invention, such as hybridoma LM609, that secretes antibody molecules having the immunospecificity(s) described hereinabove.

The culture is maintained under conditions and for a time period sufficient for the hybridoma to secrete the antibody molecules into the medium. The antibody molecule-containing medium is then collected. The antibody molecules can then be further isolated by well known techniques.

Media useful for the preparation of these compositions are both well known in the art and commercially available and include synthetic culture media, inbred mice and the like. An exemplary synthetic medium is Dulbecco's minimal essential medium [DMEM; Dulbecco et al., Virol. 8:396 (1959)] supplemented with 4.5 gm/l glucose, 20 mm glutamine, and 20% fetal calf serum. An exemplary inbred mouse strain is the Balb/c.

The monoclonal antibodies produced by the above method can be used, for example, in diagnostic and therapeutic modalities wherein formation of an ECr-containing immunoreaction product is desired.

C. Hybridomas

A hybridoma of the present invention is characterized as producing a monoclonal antibody (Mab) capable of immunoreacting with ECr. A preferred hybridoma produces a Mab that is substantially free of antibody molecules that immunoreact with VNr or FNr. More preferably a hybridoma of the present invention

is characterized as producing a Mab capable of immunoreacting with HUVE cells, BAE cells, CEE cells and the like and thereby inhibiting their adhesion to vitronectin, fibrinogen, von Willebrand factor or a hexapeptide having the amino acid residue sequence GRGDSP. Still more preferably a hybridoma is characterized herein as being capable of immunoreaction with a complex-dependent determinant present on ECr.

Methods for producing hybridomas that secrete antibody molecules having a desired immunospecificity, i.e., having the ability to immunoreact with an identifiable epitope on a particular protein, are well known in the art. Particularly applicable is the hybridoma technology described by Galfre et al., Meth. Enzymol., 73:3-46 (1981), which description is incorporated herein by reference. Briefly, to form the hybridoma from which a monoclonal antibody of this invention can be produced, a myeloma or other self-perpetuating cell line is fused with lymphocytes obtained from the spleen of a mammal hyperimmunized with an immunologically effective amount of ECr.

It is preferred that the myeloma cell line be from the same species as the lymphocytes. Typically, a mouse of the strain 129 Glx^+ is the preferred mammal. Suitable mouse myelomas for use in the present invention include the hypoxanthine-aminopterin-thymidine-sensitive (HAT) cell lines P3X63-Ag8.653, and Sp2/0-Ag14 that are available from the American Type Culture Collection, Rockville, MD, under the designations CRL 1580 and CRF 1581, respectively.

Splenocytes are typically fused with myeloma cells using polyethylene glycol (PEG) 2000. Fused

hybrids are selected by their sensitivity to HAT. Hybridomas capable of producing a monoclonal antibody of this invention are identified by the screening methods described in Example 2. First, the culture supernatants from HAT selected hybridomas are screened in an ELISA assay for the presence of antibody molecules capable of immunoreaction with M21 cells. Hybridomas that produced antibody molecules with the ability to immunologically bind M21 cells were retained and further screened for their ability to produce antibody molecules that could inhibit ECr-containing cells from adhering to vitronectin using the adhesion assay in Example 5. Those hybridomas that produced adhesion inhibiting antibody molecules in that assay were retained as hybridomas of the present invention.

Preferred hybridomas of the present invention are further screened for the ability to immunoreact with a complex-dependent determinant present on the ECr heterodimer complex using the method described in Example 12.

A preferred hybridoma is hybridoma LM609 that has been deposited pursuant to Budapest Treaty requirements with the ATCC on September 15, 1987, and was assigned accession number HB 9537.

D. Therapeutic Methods and Compositions

A monoclonal antibody of the present invention can be used to inhibit the binding interaction of ECr with vitronectin, fibrinogen and von Willebrand factor in vivo.

For instance, a monoclonal antibody of the present invention contains antibody molecules that immunoreact with ECr to form an antibody molecule-ECr complex, present on the endothelial cell-surface, so that the cell does not bind vitronectin, fibrinogen or

von Willebrand factor. Thus, a monoclonal antibody of the present invention can be used in a pharmaceutically acceptable composition that, when administered intravenously in an effective amount, is capable of inhibiting the ability of ECr molecules present on the surface of tumor cells and blood vessel-forming endothelial cells to bind vitronectin, fibrinogen and von Willebrand factor present as part of the subendothelial matrix. That inhibition is believed to result in a decreased amount of attachment by cells containing the ECr to tissues in the body containing exposed vitronectin, fibrinogen and von Willebrand factor. Thus, in vivo administration of a monoclonal antibody composition of the present invention can be used to inhibit tumor growth because such growth depends on cell attachment.

The monoclonal antibody compositions administered take the form of solutions or suspensions and contain 0.1-95% of active ingredient, preferably 25-70%.

The preparation of therapeutic compositions which contain antibody molecules as active ingredients is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting

or emulsifying agents, pH buffering agents which enhance the effectiveness of the active ingredient.

A monoclonal antibody can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The therapeutic antibody molecule-containing compositions are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosages, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient without adverse side affects, and degree of inhibition of interaction desired between ECr and the appropriate matrix protein. Precise amounts of active

ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges are of the order of 0.1 to 10 milligrams, preferably one to several milligrams, of active ingredient per kilogram bodyweight of individual per day and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated.

E. Diagnostic Systems

A diagnostic system in kit form of the present invention includes, in an amount sufficient for at least one assay, a monoclonal antibody of the present invention, as a separately packaged reagent. Preferably, the anti-ECr antibody molecules present in the monoclonal antibody are those produced by hybridoma LM609. Instructions for use of the packaged reagent are also typically included.

Methods for optimizing the amount of an antibody used in a given diagnostic method are well known in the art. In vitro methods typically require in the range of from about one microgram to several milligrams of antibody protein. In vivo methods typically require in the range of from about one milligram to several hundred milligrams per kilogram (kg) bodyweight of individual.

"Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to

be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like.

In preferred embodiments, a diagnostic system of the present invention further includes a label or indicating means capable of signaling the formation of a complex produced by immunoreaction of ECr with a monoclonal antibody of this invention. Thus, in preferred diagnostic systems, the anti-ECr antibody molecules present in a monoclonal antibody composition of the present are linked to an indicating means.

The word "complex" as used herein refers to the product of a specific binding reaction such as an antibody-antigen or receptor-ligand reaction. Exemplary complexes are immunoreaction products.

As used herein, the terms "label" and "indicating means" in their various grammatical forms refer to single atoms and molecules that are either directly or indirectly involved in the production of a detectable signal to indicate the presence of a complex. "In vivo" labels or indicating means are those useful within the body of a subject and include ^{111}In, ^{99}Tc, ^{67}Ga, ^{186}Re and ^{132}I. Any label or indicating means can be linked to or incorporated in the antibody molecule that is part of a monoclonal antibody composition of the present invention, or used separately, and those atoms or molecules can be used alone or in conjunction with additional reagents. Such labels are themselves well-known in clinical diagnostic chemistry and constitute a part of this invention only insofar as they are utilized with otherwise novel proteins methods and/or systems.

The labeling means can be a fluorescent labeling agent that chemically binds to antibodies or

antigens without denaturing them to form a fluorochrome (dye) that is a useful immunofluorescent tracer. Suitable fluorescent labeling agents are fluorochromes such as fluorescein isocyanate (FIC), fluorescein isothiocyante (FITC), 5-dimethylamine-1-naphthalenesulfonyl chloride (DANSC), tetramethylrhodamine isothiocyanate (TRITC), lissamine, rhodamine 8200 sulphonyl chloride (RB 200 SC) and the like. A description of immunofluorescence analysis techniques is found in DeLuca, "Immunofluorescence Analysis", in Antibody As a Tool, Marchalonis, et al., eds., John Wiley & Sons, Ltd., pp. 189-231 (1982), which is incorporated herein by reference.

In preferred embodiments, the indicating group is an enzyme, such as horseradish peroxidase (HRP), glucose oxidase, or the like. In such cases where the principal indicating group is an enzyme such as HRP or glucose oxidase, additional reagents are required to visualize the fact that a paratopic molecule-antigen complex (immunoreactant) has formed. Such additional reagents for HRP include hydrogen peroxide and an oxidation dye precursor such as diaminobenzidine. An additional reagent useful with glucose oxidase is 2,2'-azino-bis-(3-ethyl-benzthiazoline-sulfonic acid) (ABTS).

Radioactive elements are also useful labeling agents and are used illustratively herein. An exemplary radio labeling agent is a radioactive element that produces gamma ray emissions. Elements which themselves emit gamma rays, such as ^{124}I, ^{125}I, ^{128}I, ^{132}I and ^{51}Cr represent one class of gamma ray emission-producing radioactive element indicating groups. Particularly preferred is ^{125}I. Another group of useful labeling means are those elements such

as ^{11}C, ^{18}F, ^{15}O and ^{13}N which themselves emit positrons. The positrons so emitted produce gamma rays upon encounters with electrons present in the animal's body. Also useful is a beta emitter, such ^{111}indium or ^{3}H.

The linking of labels to, i.e., labeling of, proteins is well known in the art. For instance, antibody molecules produced by a hybridoma can be labeled by metabolic incorporation of radioisotope-containing amino acids provided as a component in the culture medium. See, for example, Galfre et al., Meth. Enzymol., 73:3-46 (1981). The techniques of protein conjugation or coupling through activated functional groups are particularly applicable. See, for example, Aurameas, et al., Scand. J. Immunol., Vol. 8 Suppl. 7:7-23 (1978), Rodwell et al., Biotech., 3:889-894 (1984), and U.S. Pat. No. 4,493,795

The diagnostic systems can also include, preferably as a separate package, a specific binding agent. A "specific binding agent" is a molecular entity capable of selectively binding a reagent species of the present invention or a complex containing such a species, but is not itself an antibody molecule composition of the present invention. Exemplary specific binding agents are second antibody molecules, complement proteins or fragments thereof, S. aureus protein A, and the like. Preferably the specific binding agent binds the reagent species when that species is present as part of a complex.

In preferred embodiments, the specific binding agent is labeled. However, when the diagnostic system includes a specific binding agent that is not labeled, the agent is typically used as an amplifying means or reagent. In these embodiments,

the labeled specific binding agent is capable of specifically binding the amplifying means when the amplifying means is bound to a reagent species-containing complex.

The diagnostic kits of the present invention can be used in an "ELISA" format to detect the presence or quantity of ECr in a body fluid sample such as serum, plasma or urine. "ELISA" refers to an enzyme-linked immunosorbent assay that employs an antibody or antigen bound to a solid phase and an enzyme-antigen or enzyme-antibody conjugate to detect and quantify the amount of an antigen or antibody present in a sample. A description of the ELISA technique is found in Chapter 22 of the 4th Edition of Basic and Clinical Immunology by D.P. Sites et al., published by Lange Medical Publications of Los Altos, CA in 1982 and in U.S. Patents No. 3,654,090; No. 3,850,752; and No. 4,016,043, which are all incorporated herein by reference.

Thus, in preferred embodiments, a monoclonal antibody of the present invention can be affixed to a solid matrix to form a solid support that comprises a package in the subject diagnostic systems.

The reagent is typically affixed to the solid matrix by adsorption from an aqueous medium although other modes of affixation, well known to those skilled in the art, can be used.

Useful solid matrices are also well known in the art. Such materials are water insoluble and include the cross-linked dextran available under the trademark SEPHADEX from Pharmacia Fine Chemicals (Piscataway, NJ); agarose; beads of polystyrene beads about 1 micron to about 5 millimeters in diameter available from Abbott Laboratories of North Chicago, IL; polyvinyl chloride, polystyrene, cross-linked

polyacrylamide, nitrocellulose- or nylon-based webs such as sheets, strips or paddles; or tubes, plates or the wells of a microtiter plate such as those made from polystyrene or polyvinylchloride.

A monoclonal antibody, labeled specific binding agent or amplifying reagent of any diagnostic system described herein can be provided in solution, as a liquid dispersion or as a substantially dry powder, e.g., in lyophilized form. Where the indicating means is an enzyme, the enzyme's substrate can also be provided in a separate package of a system. A solid support such as the before-described microtiter plate and one or more buffers can also be included as separately packaged elements in this diagnostic assay system.

The packages discussed herein in relation to diagnostic systems are those customarily utilized in diagnostic systems. Such packages include glass and plastic (e.g., polyethylene, polypropylene and polycarbonate) bottles, vials, plastic and plastic-foil laminated envelopes and the like.

F. Assay Methods

The present invention contemplates any method that results in detecting ECr by producing an immunoreaction product using a monoclonal antibody of the present invention. Preferably, the anti-ECr antibody molecules used are those produced by hybridoma LM609. Those skilled in the art will understand that there are numerous well known clinical diagnostic chemistry procedures that can be utilized to form those products. Thus, while exemplary assay methods are described herein, the invention is not so limited.

1. Detection of ECr In Vivo

A method for detecting the presence of

ECr contained within the body of a subject is contemplated. A diagnostically effective amount of a monoclonal antibody of the present invention, wherein the anti-ECr antibody molecules present are linked to a in vivo indicating means, is intravenously administered into the subject. In preferred embodiments the labeled antibodies are those that are secreted by hybridoma LM609.

A diagnostically effective amount of a monoclonal antibody is typically an amount of about 5 to about 1000 milligrams (mg) of the linked antibody in a pharmaceutically acceptable excipient per kg bodyweight of recipient, and more preferably about 10 to about 20 mg/kg. The amount of monoclonal antibody utilized depends, inter alia, upon the condition of the subject and the type of indicating means linked to the antibody.

The subject is then maintained for a predetermined time period sufficient for the labeled antibody molecules to immunoreact with any ECr that is present and form an immunoreaction product, and preferably for an additional time period sufficient for a substantial amount of any non-reacted antibody molecules to clear the body. The subject is then assayed for the presence and preferably the location of any labeled product that formed.

2. Detection of ECr in a Body Sample

Various heterogeneous and homogeneous assay protocols can be employed, either competitive or non-competitive, for detecting the presence and preferably the amount of ECr in a body fluid sample such as blood or in a whole cell preparation such as a tissue section. For example, a preparation of tissue biopsy is prepared for immunofluorescence analysis by fixation and admixed with a monoclonal antibody of

this invention containing anti-ECr antibody molecules labeled with glucose-oxidase. The immunoreaction admixture thus formed is maintained under biological assay conditions for a time period sufficient for any ECr present in the sample to immunoreact with the labeled antibody molecules to form a labeled immunoreaction product. The labeled immunoreaction products are then separated from the non-reacted labeled antibody molecules, typically by washing the fixed sample sufficient to rinse away all non-reacted labeled antibody molecules without removing significant amounts of immunoreacted antibody present in the sample. The amount of labeled immunoreaction product formed is then assayed.

Biological assay conditions are those that maintain the biological activity of the antibody molecules of this invention and ECr sought to be assayed. Those conditions include a temperature range of about 4 degrees C to about 45 degrees C, preferably about 37 degrees C, a pH value range of about 5 to about 9, preferably about 7 and an ionic strength varying from that of distilled water to that of about one molar sodium chloride, preferably about that of physiological saline. Methods for optimizing such conditions are well known in the art.

Examples

The following examples are intended to illustrate, but not limit, the present invention.

1. Isolation of ECr from M21 Melanoma Cells

M21 human melanoma cells were kindly provided by Dr. D.L. Morton (University of California at Los Angeles, CA). The isolation of the ECr was performed as previously described by Cheresh et al., J. Biol. Chem., 262:4 1434-1437 (1987). Briefly, M21

cells were grown as a suspension culture in growth medium [RPMI 1640 containing 2mM L-glutamine and 50 micrograms per milliliter (mg/ml) gentamicin sulfate; all from GIBCO Laboratories, Grand Island, NY] containing 10 percent fetal calf serum (FCS) at 37 degrees C in 7.5% CO_2/95% air.

To methionine-free growth medium (GIBCO) containing 3.5 X 10^7 M21 cells per milliliter (ml) was admixed ^{35}S-methionine [ICN Biochemicals, Irvine, CA; specific activity 1140 curies per millimole (Ci/mmole)] to a concentration of 1-2 millicuries (mCi) per ml. The admixture was then maintained at 37°C for 4 hours after which the cells contained therein were harvested by centrifugation at 400 x g to produce a packed cell pellet. Five ml of the packed pellet were admixed with 5 ml of extraction buffer [phosphate buffered saline (PBS) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 mM octyl beta-D-glucopyranoside (octylglucoside; Behring Diagnostics, La Jolla, CA), 3 mM phenylmethyl sulfonyl flouride (PMSF)]. The extraction admixture was maintained for 15 minutes at 4 degrees C and then centrifuged at 25,000 x g for 30 minutes to form a supernate that was recovered to form a ^{35}S-labeled M21 cell extract.

An affinity column comprising the synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK) covalently linked to Sepharose beads was prepared using cyanogen bromide-activated Sepharose (Pharmacia Inc., Piscataway, NJ) according to the manufacturer's instructions to form GRGDSPK-Sepharose having 20 mg of peptide per ml of packed beads. The peptide GRGDSPK utilized herein, in addition to peptides Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP), were provided by Dr. M.D. Pierschbacher (La Jolla Cancer Research Foundation, La Jolla, CA) after

having been synthesized by Peninsula Laboratories (San Carlos, CA).

Five ml of ^{35}S-labeled M21 cell extract was admixed with 5 ml of packed GRGDSPK-Sepharose beads and the resulting admixture was maintained at 4 degrees C for 12-1 8 hours to allow M21 ECr to bind to the GRGDSPK-Sepharose. The GRGDSPK-Sepharose was then transferred to a column containing a scintered glass support, and washed with 25 ml of extraction buffer containing 50 mM octylglucoside. Thereafter extraction buffer containing 50 mM octylglucoside and 1 mg/ml of the peptide GRGDSP was added to the column and 1.25 ml fractions were collected as the added buffer came off (eluted from) the column to form eluted fractions.

Fifty microliters (ul) of each eluted fraction was then analyzed by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% acrylamide gels containing 3% SDS and 5% 2-mercaptoethanol according to the methods of Laemmli, Nature, 227:680-685 (1970). Proteins contained in the eluted fractions so analyzed were visualized on the SDS-PAGE gels by autoradiography. The apparent molecular weight (Mr) of the visualized proteins was determined by comparison to protein molecular weight markers also analyzed on the gel that include myosin, 200; beta-galactosidase, 116; phosphorylase B, 94; bovine serum albumin, 67; ovalbumin, 43; and carbonic anhydrase, 30; all molecular weights in units of one thousand daltons.

Fractions containing eluted proteins that exhibited molecular weights of approximately 130 and 105 kilodaltons (kDa) were collected and pooled to form an isolated ECr-containing solution.

2. Preparation of Hybridomas Using ECr Isolated from Melanoma Cells

a. Preparation of Immunogen

Two ml of isolated ECr-containing solution prepared using unlabeled M21 cells in the procedure described in Example 1 were admixed with 1 ml of lentil lectin-Sepharose (Pharmacia) and maintained for 2 hours at room temperature. Thereafter the admixture was transferred to a column containing a scintered glass support and the retained Sepharose beads were washed with about 20 mls of extraction buffer to form ECr-bound Sepharose beads.

b. Hybridoma Formation

Hybridomas that secrete antibody molecules that immunoreact with ECr were prepared by immunization with ECr-bound Sepharose beads prepared in Example 2a. One hundred microliters (ul) of a suspension containing 50 ul PBS and 50 ul of ECr-bound Sepharose beads were injected intraperitoneally (ip) once every week for a total of 6 weeks into Balb/c mice. Three days after the last injection, the splenocytes of these mice were removed, a suspension of the splenocytes was made, and the splenocytes were fused with a non-secreting murine myeloma cell line Sp2/0 subclone M5 in the presence of a cell fusion promoter (polyethylene glycol 2000, Boehringer Mannheim Biochemicals, Indianapolis, IN) to form hybridomas using the standard hybridoma technology described by Galfre et al., Meth. Enzymol., 73:3-46 (1981). The resulting hybridomas were selected by growth in a culture medium that does not support the growth of unfused myeloma cells and contains hypoxanthine, aminopterin and thymidine (HAT), and were thereafter subcloned using limiting dilution and culturing in separate containers. The resulting

culture supernatant in each container was then evaluated for the presence of antibody molecules that immunoreact with (bind to) ECr contained on intact M21 cells using the ELISA assay described in Example 2c.

c. Screening Hybridomas by M21 Cell ELISA

M21 cells, cultured as described in Example 1, were washed twice in normal saline and plated in a flat-bottom polyvinyl microtiter plate (Dynatech, Chantilly, VA) at 5 X 10^4 cells per well in 50 ul in growth medium containing 10% FCS. Thereafter the plates were maintained at 37 degrees C for about 12-18 hours to dry the plates.

Dried plates were rehydrated by two washes each comprising first the addition of wash buffer [10 mM PBS, pH 7.4, 0.1% Tween 20 (polyoxyethylenesorbitan monolaurate) 0.02% thimerosal (sodium ethylmercurithiosalicylate)], then the maintenance of the plates at room temperature for two minutes, and finally the removal of the liquid by inverting the plates. Antibody molecules contained in hybridoma supernatants, prepared in Example 2b and further isolated using protein A Sepharose as described in Example 3, were diluted in diluent buffer [1:10(v/v) in wash buffer containing 0.1% BSA (bovine serum albumin)] and 50 ul thereof was admixed into each well of the rehydrated plates to form immunoreaction admixtures. The plates were maintained for one hour at 4 degrees C on a gyroshaker, emptied of their liquid contents by inversion and shaking, and then washed twice as described above to isolate solid-phase bound immunoreaction products. Afterwards, 50 ul of a solution containing peroxidase-linked goat anti-mouse Ig (Tago, Burlingame, CA) diluted 1:1000 (v/v) in diluent were admixed into each well of the

plates and maintained for 1 hour at 4 degrees C to allow formation of labeled immunoreaction products. The plates were again emptied by inversion and shaking, washed twice as described above, and 50 ul of freshly prepared chromogenic substrate solution [400 ug/ml O-phenylene diamine (Sigma Chemical Co., St. Louis, MO), 80 mM citrate-phosphate buffer, pH 5.0, 0.012% (v/v) H_2O_2 (Eastman Kodak, Rochester, NY)] was admixed into each well. The plates were then maintained in the dark for 30 minutes after which time 15 ul of 4N H_2SO_4 was admixed into each well. The optical absorbance at 492 nanometers (A_{492}) of the resulting solutions in each well was then measured with a Multiscan ELISA plate reader (Flow Laboratories, McLean, VA). Antibody molecules produced by hybridoma cultures assayed in such a manner were considered positive where the optical density at 492 nanometers ($O.D._{492}$) exceeded 0.1.

The above described hybridoma production and isolation procedure resulted in the production of two monoclonal hybridoma cell lines that each secreted anti-ECr antibody molecules.

Hybridomas LM142 and LM609 were then further screened for the ability to secrete antibody molecules that could inhibit adhesion of M21 melanoma cells to vitronectin using the adhesion assay described in Example 5. Hybridomas of the present invention are those which secrete anti-ECr antibody molecules capable of reducing by greater than 50% the amount of M21 cells that attach when compared to the amount that attach in the presence of a heterologous monoclonal antibody (negative control).

3. Monoclonal Antibody Production

A monoclonal antibody of the present invention is prepared from a hybridoma culture

supernatant by separating the supernatant from the hybridoma cells to form a cell-free antibody molecule-containing solution. Alternatively, a monoclonal antibody can be produced from ascites by introducing, as by injection, a hybridoma prepared in Example 2 into the peritoneal cavity of a mammal such as a mouse and later harvesting the resulting peritoneal exudate (ascites tumor fluid) from the mouse by well known techniques.

Anti-ECr antibody molecules present in recovered antisera, ascites fluid or hybridoma culture supernates can then be further isolated by affinity chromatography using protein-A Sepharose (Bio-Rad Laboratories, Richmond, CA) according to the methods recommended by the manufacturer with the exception that the elution buffer should be adjusted to pH 5.0.

The protein concentration of the resulting protein-A Sepharose isolated antibody containing-solutions was determmined typically using the Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA) according to the manufacturer's instructions.

4. Immunoprecipitation Analysis of ECr from M21 Melanoma Cells

ECr analyzed by immunoprecipitation was prepared as follows from ^{35}S-labeled M21 cells.

M21 melanoma cells described in Example 1 were maintained at a concentration of 3.5×10^7 cells/ml for 15 minutes at 37°C in methionine-free growth medium (GIBCO) containing 10% FCS. Subsequently, ^{35}S-methionine was admixed with the cells to a concentration of 0.5-1mCi/ml. After maintaining the cells for 10 minutes 5×10^6 cells were then removed and designated as zero time point cells. The remaining cells were centrifuged at 400 x g to separate them from free ^{35}S-methionine and the

resulting pellet was washed four times in growth medium containing 4 mM unlabeled L-methionine. After the final wash, 5 X 10^6 labeled cells were resuspended at about 3.5 X 10^7 cells/ml in growth medium containing 10% FCS and maintained at 37°C for 8 hours. The ^{35}S-methionine-labeled cells were then centrifuged at 400 x g, and the resulting pellet was washed as before and recentrifuged to form a labeled M21 cell pellet.

Lysates were then prepared by resuspending the labeled M21 cell pellet in RIPA lysis buffer (0.1 M Tris-HCl [Tris (hydroxymethyl) amino methane hydrochloride], pH 7.2, 0.15 M NaCl, 1% (w/v) deoxycholate, 1% (w/v) Nonident P-40 (an octylphenol ethylene oxide condensate containing an average of 9 moles ethylene oxide per phenol; Sigma), 0.1% (w/v) SDS, 10% (w/v) aprotinin (Sigma, product No. A6279), 2 mM PMSF) at a concentration of 10^6 cells/ml. The resulting lysates were maintained for 15 min at 0 °C to ensure maximum cell lysis and then were subjected to ultracentrifugation at 35,000 rpm in a Beckman Ti50 rotor (Beckman Instruments, Palo Alto, CA) for 45 minutes to clear cell debris and form a ^{35}S-labeled cell lysate.

Ten mg of antibody protein isolated using protein A-Sepharose as described in Example 3 were covalently coupled to 1 ml of cyanogen bromide-activated Sepharose (Pharmacia) according to the manufacturer's instructions to form immunoadsorbant beads. A suspension containing 10 ul of packed immunoadsorbant beads in 100 ul RIPA lysis buffer was admixed with 1 ml clear labeled lysate to form an immunoreaction admixture. The admixture was then maintained overnight (16-18 hours) at 4°C to allow formation of M21 ECr-immunoadsorbant bead complexes

containing an immunoreaction product. The beads were then centrifuged at 12,000 x g for about ten seconds at 20 degrees C, and the resulting pellet was recovered and washed five times in 1 ml volumes of phosphate-buffered saline, pH 7.2 containing 0.1% ovalbumin and 0.5% Tween 20. The immunoreaction product was solubilized by boiling for 3 minutes in 100 ul of sample buffer (30 mM Tris-HCl, pH 6.8, 10% glycerol and bromphenol blue) and analyzed by SDS-PAGE under reducing conditions on 7.5% gels according to the methods of Laemmli, Nature, 227:680-685 (1970). The labeled protein bands were visualized on SDS-PAGE gels by autoflourography. The apparent molecular weights (Mr) of the visualized proteins were determined as described in Example 1. As shown in Figure 1, antibody molecules produced by hybridomas LM142 and LM609 immunoreacted with the ^{35}S-labeled ECr of M21 cells containing alpha and beta subunits of about 130 and 105 kilodaltons (kDa), respectively.

5. M21 Cell Adhesion Assay

The M21 cells were grown in suspension culture in RPMI 1640 growth medium containing 10% fetal calf serum (FCS) at 37°C with 7.5% CO_2/92.5% air. These cells were metabolically labeled in leucine-free growth media containing 50 microCuries (uCi)/ml ^{3}H leucine (ICN, Irvine, CA) for 72 hours at 37°C. Thereafter labeled cells were washed by centrifugation in growth medium containing 1% FCS to remove unincorporated radiolabel forming ^{3}H-leucine-labeled M21 cells.

Approximately 5 X 10^3 ^{3}H-leucine labeled M21 cells were resuspended in 100 ul growth medium containing protein A-Sepharose purified Mab LM609 (Example 3) at a concentration of 0.5 mg/ml to form an immunoreaction admixture. As controls, labeled cells

were resuspended and maintained in growth medium alone, or a heterologous antibody was used. The admixture was then maintained for 1 hour at 4 degrees C to allow formation of Mab LM609-M21 cell immunoreaction products. The cells were then washed by two cycles of low speed centrifugation at 400 x g and resuspension of the resulting pellet using growth medium containing 1% FCS. Thereafter the cells were resuspended in growth medium to form antibody treated M21 cells.

The wells of polystyrene microtiter plates (96-well; Flow Laboratories, McLean, VA) were coated with matrix proteins (adhesion substrates) by admixing solutions of PBS (pH 7.2) containing 5-20 ug/ml of an adhesion substrate to a well. The wells were maintained overnight at 25 degrees C to allow adsorption of the proteins onto the wells. Matrix proteins used included fibrinogen, provided by Dr. E. Plow (Research Institute of Scripps Clinic, La Jolla, CA, hereinafter RISC); von Willebrand factor and size fractionated von Willebrand factor, wherein pool I contains large multimers of greater than 5 X 10^3 kDa and pool III contains multimers from 2-5 X 10^3 kDa all being provided by Drs. Z. Ruggeri and T. Zimmerman (RISC) ; collagen type I and IV (Collaborative Research Inc., Bedford, MA); and human fibronectin, vitronectin and laminin were provided by Dr. M. Pierschbacher (La Jolla Cancer Research Foundation, La Jolla, CA hereinafter LCRF).

Where the adhesion substrate utilized was a synthetic peptide, the peptide was coated onto plates by a different procedure. To the wells of a 96 well polystyrene microtiter plate (Flow Laboratories) was added approximately 100 ul of a PBS solution containing human gamma globulin Fraction II (20 ug/ml;

Sigma Chemical Co., St. Louis, MO), and the plates were then maintained for 4 hours at room temperature. Thereafter the wells were emptied by inversion, washed three times with 100 ul of PBS, emptied again, filled with 50 ul of a solution containing SPDP [3-(2-pyridyldithio)proprionic acid N-hydroxy-succinimide ester] (10 ug/ml in PBS) and maintained at room temperature for 30 minutes to form SPDP activated plates. The wells of a SPDP activated plate were then washed twice as before, filled with 50 ul of a PBS solution containing the peptide GRGDSPC (Dr. Pierschbacher, LCRF) at a concentration of 15 ug per ml and maintained at room temperature for 2 hours.

The plates were then inverted to remove the peptide coating solution from the wells. Residual uncoated sites were blocked by admixing a solution of PBS containing 1% BSA into each well and maintaining the wells for 1 hour at room temperature to form peptide (adhesion substrate) coated wells.

Fifty ul of growth medium containing 5 X 10^3 antibody treated M21 cells were admixed in an adhesion substrate-coated well to form an adhesion-reaction admixture. The admixtures were maintained at 37 degrees C in a humidified incubator for the times indicated in Figure 2, at which point the plates were inverted to remove growth medium and non-adhered cells. All wells were then washed twice with 150 ml PBS (pH 7.2) to assure removal of unattached cells. The remaining attached cells were harvested by adding 100 ul of Trypsin/EDTA (1X; Gibco Laboratories, Grand Island, NY) to each well, incubating the cells at 37 degrees C for 30 minutes and then removing the cells and collecting them on glass fiber filters using a Skatron automated cell harvester (Skatron Instruments, Sterling, VA) according to instructions provided by

the manufacturer. The fiber filter disks were placed into vials containing 5 ml of liquid scintillation cocktail and the amount of radioactive label present determined as counts per minute in a liquid scintillation counter. Results were expressed as the total number of cells (counts per minute) bound at the designated time points.

As shown in Figure 2, Mab LM609 immunoreacted with M21 cells and significantly inhibited their adhesion (attachment) to von Willebrand factor, fibrinogen and the peptide GRGDSPC yet had little or no effect on attachment to fibronectin, and collagen types I and IV.

6. Isolation of Human Endothelial Cell ECr

ECr from metabolically labeled human umbilical vein endothelial (HUVE) cells was prepared by first isolating HUVE cells from fresh human umbilical veins and then propagating the isolated cells as described by Jaffe et al., J. Clin. Invest., 52:2745-2756 (1973), which is incorporated herein by reference. HUVE cells were then labeled by the addition of 2 microCuries of ^{35}S-methionine (1295 Ci/millimole, New England Nuclear) to a T-150 tissue culture flask (Corning Glass, Corning, NY) containing the cells at semi-confluency in 20 ml of growth medium (RPMI 1640 containing 10% FCS). The culture was then maintained for 72 hours at 37^{o}C to allow formation of ^{35}S-labeled HUVE cells.

The labeled HUVE cells were then harvested by adding sufficient versene [10 mM ethylene-diaminetetraacetic acid (EDTA) in PBS] to cover the monolayer of labeled cells, maintaining the monolayer at 37 degrees for about 10 minutes or until the monolayer cells become detached from the culture flask

and then collecting the detached cells. The detached cells were then centrifuged in a clinical centrifuge at 1500 rpm for about 5 minutes to form a labeled HUVE cell pellet.

The resulting pellet was admixed with an equal volume of extraction buffer (PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 mM octylglucoside, and 3 mM PMSF). The admixture was maintained for 15 minutes at 4°C, centrifuged at 25,000 x g for 30 minutes and the resulting supernate was then recovered to form a labeled HUVE cell extract.

The labeled HUVE cell extract was then admixed with an equal volume of the GRGDSPK-Sepharose beads prepared in Example 1. The ECr contained in the extract was then isolated and analyses by SDS-PAGE as described in Example 1. Fractions containing eluted proteins that exhibited relative molecular weights of approximately 135 kDa and 115 kDa were individually retained as purified ECr.

Figure 3 shows the GRGDSPK-Sepharose column elution profile of the labeled HUVE cell extract. When the non-specific peptide GRGESP was used in the elution buffer, no appreciable ^{35}S-labeled ECr protein was eluted. However, when peptide GRGDSP was added to the elution buffer, a single peak, comprised of fractions 14-18, was eluted containing the 135 kDa and 115 kDa ECr subunit proteins.

7. Immunoprecipitation of Human Endothelial Cell ECr

Fractions 14-16 of purified ECr from Example 6 were pooled and 50 ul of that pool was admixed with an equal volume of 2X RIPA lysis buffer (0.1 M Tris-HCl, pH 7.2, 0.15 M NaCl, 1% (w/v) deoxycholate, 1% (w/v) Nonident P-40, 0.1% (w/v) SDS, 1% (w/v) aprotinin, 2 mM PMSF). The ability of the resulting

admixture to immunoreact with either Mab LM609 or Mab LM142 was then determined by immunoprecipitation analysis as described in Example 4. The results of those analyses, shown in Figure 4, indicate that both Mab LM609 and Mab LM142 immunoreacted with the purified endothelial ECr heterodimer complex comprised of alpha and beta subunits of about 135 kDa and 115 kDa, respectively.

Similarly, bovine aortic endothelial (BAE) cells were used utilized as a bovine source of ECr for immunoprecipitation analysis. BAE cells were isolated from the aorta of cows by the method of Booyse et al., Thromb. Diathes. Haemorrh., 34:825 (1975), whose teachings are incorporated herein by reference. Isolated BAE cells were cultured in modified Eagle's medium supplemented with 10 percent FCS as described in Levin et al., Thromb. Res., 15:869 (1979).

Cultured BAE cells were labeled with ^{35}S-methionine and harvested as in Example 6 to form a labeled BAE cell pellet. Lysate preparation and immunoprecipitation analysis were then performed on the labeled BAE cell pellet as described in Example 4. Immunoadsorbants prepared from Mab LM609 immunoreacted with ECr present in BAE lysates as indicated by the presence in the immunoreaction products of proteins having molecular weights of about 135 kDa, i.e., molecular weights corresponding to the alpha and beta subunits, respectively, of ECr detected in HUVE cells.

8. Human Endothelial Cell Adhesion Assay

Human umbilical vein endothelial (HUVE) cells were grown in monolayer cultures as described in Example 6. The HUVE cell monolayers were metabolically labeled by maintaining cells for 72 hours at 37°C in growth medium containing 50 uCi/ml ^{3}H-leucine. The labeled cells were detached from the

monolayer culture flask using a Trypsin/EDTA solution as is well known and then washed in growth medium containing 1% FCS by low speed centrifugation in the same medium to form ^{3}H-leucine labeled HUVE cells.

The ^{3}H-leucine labeled HUVE cells were analyses in the adhesion assay in the same manner as the ^{3}H-leucine labeled M21 cells were analyzed as described in Example 5. Those cells which adhered to adhesion substrate coated wells were then harvested and the radioactivity contained therein determined as described in Example 5.

As shown in Figure 5, Mab LM609 but not heterologous Mab W6/32, immunoreacted with HUVE cells and significantly inhibited their adhesion (attachment) to fibrinogen, vitronectin, von Willebrand factor and the peptide GRGDSPC, yet had little or no effect on attachment to fibronectin, collagen type IV, or laminin.

9. Partial Amino Acid Sequence of ECr of M21 Melanoma Cells

ECr was isolated from M21 melanoma cells as described in Example 1. The resulting isolated ECr-containing solutions were concentrated approximately 22 fold using a Centricon 30 microconcentrator (Amicon Corp, Danvers, MA; product No. 4209) according to the manufacturer's instructions using 0.05% SDS in HPLC grade water. The resulting concentrated ECr-containing solution was subjected to amino acid sequence analysis using an Applied Biosystems model 470 sequenator equipped with on-line PTH analysis using sequencing program O3RPTH, all according to the methods of and with materials provided by the manufacturer. (Applied Biosystems, Inc., Foster City, CA).

Due to the presence of an alpha and beta subunit in the concentrated ECr-containing solution, each residue analyzed at a given position yielded two different amino acids. The alpha subunit sequence of ECr was obtained by "subtracting" from the amino acid residues found at each position in the residues that corresponded to the known beta subunit sequences of VNr and GPIIb-IIIa.

The partial amino acid sequence of ECr is shown in Figure 6. A comparison of the partial amino acid sequence of the alpha and beta subunits of ECr to the respective subunits of vitronectin receptor or GpIIb-IIIa is also presented in Figure 6. This initial and partial amino acid sequence analysis of the amino terminus indicates that ECr is similar but not identical to VNr or GpIIb-IIIa.

10. Detection of ECr on the Surface of a Variety of Tumor Cells

The presence of ECr and antigenically related receptors on the surface of cells was analyzed for a large variety of tumor cell types using the ELISA assay described in Example 2c.

The monoclonal antibodies used in this analysis include LM142 and LM609, prepared in Example 2; LM534 provided by Dr. Cheresh (SCRF) that is directed to the FNr beta subunit; AP3, provided by Dr. Peter Newman (The Blood Center of Southeastern Wisconsin, (Milwaukee, WI), that is directed to the platelet glycoprotein IIIa [GpIIIa; Newman et al., Blood, 65:227-232 (1985)]; and W6/32, provided by Dr. Peter Parham (Stanford University, Palo Alto, CA), that is reactive with a framework determinant on human Class I histocompatibility antigens [Parham et al., J. Immunol., 123:342 (1979)].

The following cell lines were used for analysis of the presence of cell surface ECr reactive with antibodies by ELISA; sources for each line are parenthesized following the cell line designation: melanoma: M21, M14, M16A and M18 (Dr. Morton, University of California at Los Angeles, hereinafter UCLA); A375P is a sub-clone of A375 (ATCC CRL 1619), and was obtained from Dr. J. Fidler (University of Texas Health Sciences Center, Houston, TX); A375MM (Dr. J. Fidler); Melur (Dr. U. Kodovsky, Dusseldorf, West Germany); WM266 and WM239a (Dr. M. Hierlyn, Wistar Inst., Philadelphia, PA); neuroblastoma: SK-N-AS (Dr. Helson, Memorial Sloan-Kettering Cancer Center, NY); SMS-KCNR, SMS-KCN, NMB-7 and IMR-6 (Dr. P. Reynolds, UCLA); LAN-1 (Dr. Seeger, UCLA); small cell lung carcinoma: SHP77 (Dr. H.K. Oie, National Cancer Institute, Bethesda, MD); H69c (ATCC HTB 119); H82 and H417 (Drs. Minna & Gadzar, NIH, Bethesda, MD); lung adenocarcinoma: UCLA-P3 (Dr. Martin, UCLA); T291 (University of California at San Diego); squamous cell lung: Bachle and squamous cell skin: SCL-I185 (Dr. J. Mitchell, University of Southern California, Pasadena); pancreatic carcinoma: Colo 357 FG and Colo 357 SG (Dr. Kajiji, Scripps Clinic, La Jolla, CA); astrocytoma/glioblastoma: U373 MG (ATCC HTB 17); U138 MG (ATCC HTB16); U87 MG (ATCC HTB 14); T-cell lymphoma: HPB-ALL; Molt-4 (ATCC CRL 1582); HSB-2 (Dr. R. Fox, Scripps Clinic and Research Foundation, La Jolla, CA); B-cell lymphoblastoid: L14 and LG2 (Dr. Gati, UCLA); 3160,; 3107, 1857 and 3098 (NIGMS Human Genetic Mutant Cell Repository, NIH, Bethesda, MD); monocytic: U 937 (ATCC CRL 1593).

Figure 7 shows that ECr or antigenically related proteins which immunoreact with LM609 are present on the surface of most of the melanoma cell

lines examined and on some other tumor cell types, albeit in lesser amounts. It is believed that all cell types exhibiting significant immunoreaction with Mab LM609 contain ECr on their surface and thus are representative targets for the therapeutic and diagnostic methods of the present invention.

11. Inhibition of Tumor Growth by In Vivo Administration of Monoclonal Antibody LM609

M21 melanoma cells were cultured as described in Example 1. Approximately 5 X 10^6 cells were harvested by centrifugation at 400 x g for 5 minutes to produce a cell pellet. The resulting pellet was resuspended in 100 ul PBS, injected subcutaneously into the earlobe of a Balb/c nude mouse (Scripps Clinic Vivarium, La Jolla, CA) and cultured therein by maintaining the mouse for 8 days to form an established tumor. On day 8 and at 2 day intervals thereafter, 100 ul of PBS as a control, or 100 ul of PBS containing 100 ug of either Mab LM609 or Mab LM142, prepared as described in Example 2, was injected intraperitoneally (i.p.) into the mouse containing the established tumor. The established tumor then was monitored in each case for further increases in size that normally accompanies the continued maintenance of the established tumor to determine the affect of in vivo administered Mab upon the growth of the tumor.

The results of administering Mab i.p. was that the tumors in those mice that received either PBS alone or LM142 were indistinguishable from one another and had each experienced rapid logarithmic growth and increase in size between days 8 and beyond. However, the tumors in those mice receiving Mab LM609 progressed at a much slower rate over that same time

period. From the above results using the exemplary antibodies Mab LM609 and Mab LM142, it is seen that Mabs having the ability to inhibit adhesion of ECr-containing cells in vitro as shown by the methods of Example 8, are also capable of inhibiting tumor growth in vivo.

12. Assay for Detection of a Monoclonal Antibody That Immunoreacts With a Complex-Dependent Determinant Present on ECr

a. Production of Control Antisera

Rabbit anti-ECr antisera is prepared by immunization with a composition containing complete Freund's adjuvant and 100 micrograms (ug) of purified ECr protein, prepared as described in Example 6. Booster injection of ECr are administered at multiple intradermal sites (typically 100 ug distributed over 4 sites) in incomplete Freund's adjuvant on a weekly schedule for an additional three months. Antisera is then harvested using well known techniques.

b. Immunoprecipitation Analysis to Detect a Complex-Dependent Monoclonal Antibody

M21 melanoma cells were obtained and cultured as described in Example 1. M21 cells were then maintained at a concentration of 3.5×10^7 cells/ml for 15 minutes at 37°C in methionine-free growth medium (GIBCO) containing 10% FCS. Subsequently, ^{35}S-methionine was admixed with the cells to a concentration of 0.5-1 mCi/ml and the culture was then maintained at 37°C for 2 minutes to form labeled M21 cells. One culture of labeled M21 cells was then retained, the labeling medium removed and the remaining cells were designated as zero timepoint labeled M21 cells. Other cultures similarly

labeled for 2 minutes were centrifuged at 12 x g for 2 minutes at 0-4°C, resuspended in Hank's balanced salt solution (HBSS; M.A. Bioproducts, Walkersville, MD) prechilled to 0-4°C and recentrifuged as before to form washed cells. The wash procedure was repeated twice and thereafter cultures were prepared by resuspending 5 x 10^6 washed cells at about 3.5 x 10^7 cells/ml in growth medium containing 10% FCS. The washed cultures were then maintained at 37°C for chase periods of 2, 5, 10, 30, 60, 120 and 240 minutes to form pulse-chase labeled cells. The labeled cells were then washed as before to remove excess label and then recentrifuged to form a labeled M21 cell pellet.

Lysates are then prepared and immunoprecipitation analysis is conducted as described in Example 4 using both the anti-ECr control antisera from Example 12a and separately a monoclonal antibody of interest. If there is a delay of about 5 minutes in the detection time of the ECr alpha subunit precursor of about 160 kilodaltons (kDa) when using a Mab of interest in comparison to the detection time when using the control antisera, that Mab is designated a complex-dependent Mab, i.e., a Mab that immunoreacts with a complex-dependent determinant.

For example, the results of an analysis for a complex-dependent Mab is shown in Figure 8. Lysates of M21 cells pulse-chase labeled as described above were analyzed by immunoprecipitation using Mab LM142 (panel A), Mab LM609 (panel B) or Mab AP3 (panel C).

Immunoprecipitation analysis using Mab LM142 shows the detection in M21 cell pulse-chase lysates of the ECr alpha subunit precursor of about 160 kDa. The alpha subunit precursor was detectable at the zero time point and was continuously detectable

throughout the chase period to 120 minutes. In comparison, Mab LM609 detects the alpha subunit precursor in those same M21 cell lysates after a chase period of about 5 minutes and throughout the remaining chase period but not at the initial zero time point. The 5 minute delay in detection indicates that Mab LM609 is not capable of immunoreaction with the alpha subunit precursor until that subunit undergoes processing to form a heterodimer complex during the time between the zero time point and the 5 minute chase time point. On the basis of the above 5 minute delay detected by immunoreaction analysis, Mab LM142 is characterized as being capable of immunoreaction with a complex-independent determinant present on the alpha subunit precursor whereas Mab LM609 immunoreacts with a complex-dependent determinant.

Immunoprecipitation analysis using Mab AP3 shows the detection in M21 cell pulse-chase lysates of both the alpha subunit precursor and the 105 kDa beta subunit protein. Both proteins appear after a chase of about 10 minutes and persist in M21 cells for at least 120 minutes. Therefore the beta subunit is present and detectable in ECr-containing cells after 10 minutes of chase when using Mabs that immunoreact with the beta subunit of ECr, such as Mab AP3.

However, the beta subunit is not detectable in the same M21 cell lysates at times before a chase period of 120 minutes when using a complex-dependent Mab, e.g., LM609, or when using an alpha subunit specific Mab, e.g., LM142. Thus a complex-dependent Mab is therefore further characterized as being incapable of detecting the beta subunit of ECr at times earlier than about 120' of chase as shown in panel B of Figure 8.

The above description of heterodimer complex formation in the absence of a detectable 105 kDa beta subunit, for example as shown in panel B at chase times of 5 to 60 minutes, can be explained by differences in incorporation of label into the intracellular pool of beta subunit proteins when compared to incorporation into a pool of alpha subunit proteins available for heterodimer complex formation. The beta subunit incorporates label within 10 minutes of chase (panel B, 10') and is thereby detectable using beta subunit specific Mab AP3. However, labeled beta subunit does not associate with alpha subunit to form a heterodimer complex until after about 120 minutes at which time the complex immunoreacts with either alpha subunit specific Mab LM142 (panel A) or complex-dependent Mab LM609 (panel B).

Prior to 120 minutes of chase, unlabeled beta subunit already present in the available pool complexes with labeled alpha subunit. Thus, any immunoreaction product formed from lysates prepared prior to 120 minutes of chase appear to contain only the alpha subunit precursor because the beta subunit is present in the product as an unlabeled species.

The determination that a Mab immunoreacts with a complex-dependent determinant can be carried out as shown herein above, or it may be similarly accomplished using other reagent materials. For example, any cell line that contains ECr may be utilized as a source of the pulse-chase labeled ECr-containing lysate. Exemplary of said ECr-containing cells are BAE cells, HUVE cells, M21 melanoma cells and A375 melanoma cells (ATCC CRL 1619). Control antisera, such as that produced in Example 12a, may be used as a source of complex-independent antibody

molecules. The control antisera, when used in immunoprecipitation analyses of the above M21 cell lysates, exhibits the temporal pattern of immunoreaction with the alpha subunit precursor that begins at the zero time point characteristic of complex-independent antibody molecules.

The foregoing specification, including the specific embodiments and examples, is intended to be illustrative of the present invention and is not to be taken as limiting. Numerous other variations and modifications can be effected without departing from the true spirit and scope of the present invention.

What Is Claimed Is:

1. A monoclonal antibody comprising antibody molecules having an antibody combining site that immunoreacts with HUVE cells and thereby inhibits the adhesion of said cells to vitronectin, fibrinogen, von Willebrand factor or the peptide GRGDSP, and wherein said antibody combining site is produced by hybridoma HB9537.

2. The monoclonal antibody of claim 1 wherein said antibody molecules are intact immunoglobulin molecules.

3. A hybridoma that produces antibody molecules that immunoreact with HUVE cells and thereby inhibits the adhesion of said cells to vitronectin, fibrinogen, von Willebrand factor or the hexapeptide GRGDSP, and wherein the antibody combining site of said antibody molecules is produced by hybridoma HB9537.

4. A method of inhibiting tumor growth in a subject comprising administering a therapeutically effective amount of a monoclonal antibody comprising antibody molecules having an antibody combining site that immunoreacts with HUVE cells and thereby inhibits the adhesion of said cells to vitronectin, fibrinogen, von Willebrand factor or the peptide GRGDSP, and wherein said antibody combining site is produced by hybridoma HB9537, said antibody molecules being dispersed in a pharmaceutically acceptable excipient.

5. The method of claim 4 wherein said antibody molecules are intact immunoglobulin molecules.

6. A method of assaying for the presence of ECr in a body sample comprising the steps of:

a) admixing said body sample with a monoclonal antibody comprising antibody molecules

having an antibody combining site that immunoreacts with ECr present in HUVE cells and thereby inhibits the adhesion of said cells to vitronectin, fibrinogen, von Willebrand factor or the peptide GRGDSP, and wherein said antibody combining site is produced by hybridoma HB9537;

b) maintaining the admixture for a time period sufficient for said antibody molecules to immunoreact with any ECr present in said sample to form an immunoreaction product; and

c) detecting the presence of any immunoreaction product formed in step b.

7. The method of claim 6 wherein said antibody molecules are intact immunoglobulin molecules.

8. A method of assaying in vivo for the presence of ECr in a subject comprising the steps of:

a) intravenously administering to said subject a diagnostically effective amount of a monoclonal antibody comprising antibody molecules having an antibody combining site that immunoreacts with ECr present on HUVE cells and thereby inhibits the adhesion of said cells to vitronectin, fibrinogen, von Willebrand factor or the peptide GRGDSP, and wherein said antibody combining site is produced by hybridoma HB9537, said antibody molecules being linked to an in vivo indicating means and dispersed in a physiologically tolerable excipient;

b) maintaining the administered subject for a predetermined time period sufficient for said antibody molecules to immunoreact with any ECr present in said subject and form an immunoreaction product; and

c) assaying for the presence of any immunoreaction product formed in step b.

9. The method of claim 8 wherein said antibody molecules are intact immunoglobulin molecules.

10. A diagnostic system in kit form for assaying for the presence of ECr in a body sample, said system including in at least one container an effective amount of (a) a monoclonal antibody comprising antibody molecules having an antibody combining site that immunoreacts with ECr present on HUVE cells and thereby inhibits the adhesion of said cells to vitronectin, fibrinogen, von Willebrand factor or the peptide GRGDSP, and wherein said antibody combining site is produced by hybridoma HB9537, and (b) an indicating means for signaling the immunoreaction of said antibody with said ECr.

11. The diagnostic system of claim 10 wherein said antibody molecules are intact immunoglobulin molecules.

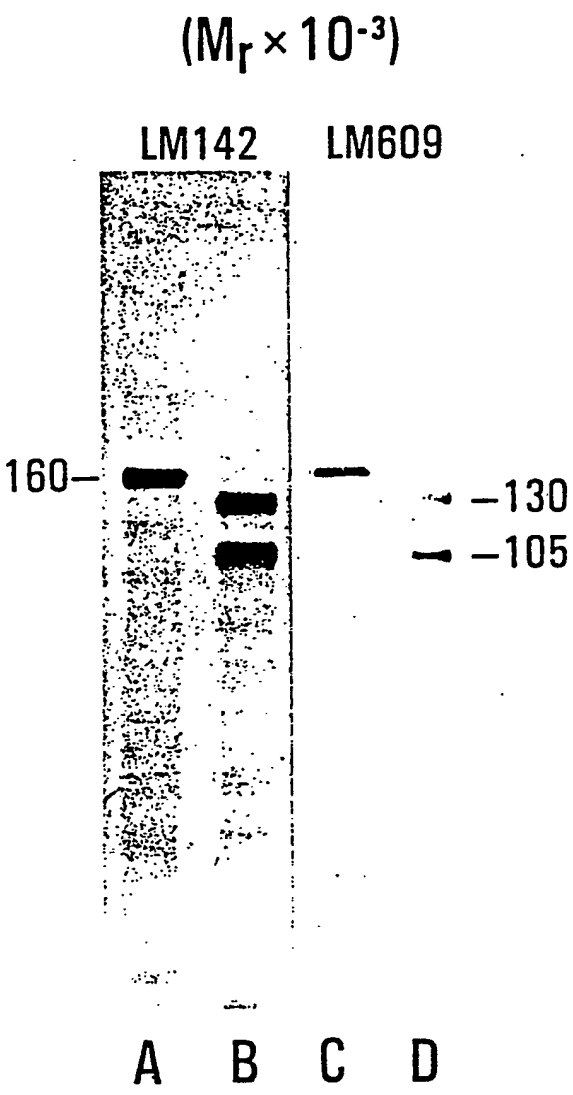

FIGURE 1

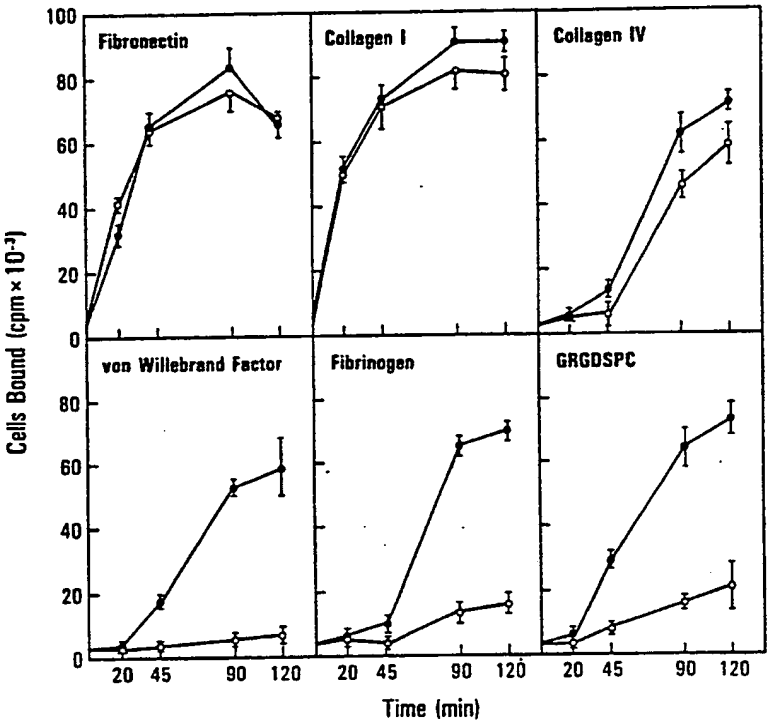
Fibronectin
Collagen I
Collagen IV
von Willebrand Factor
Fibrinogen
GRGDSPC
Cells Bound (cpm × 10⁻³)
100
80
60
40
20
0
20 45 90 120
Time (min)

FIGURE 2

AFFINITY PURIFICATION OF RGD-DIRECTED
RECEPTOR FROM HUMAN ENDOTHELIAL CELLS

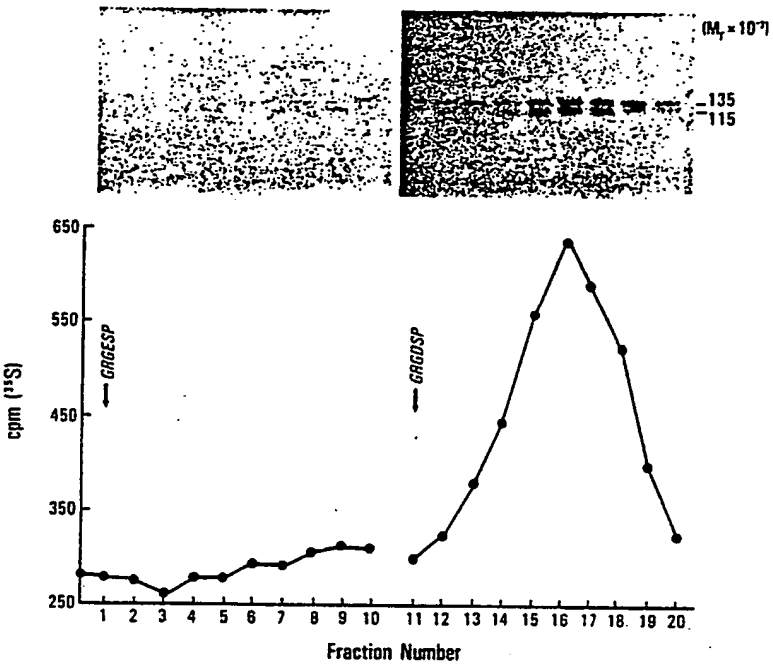

FIGURE 3

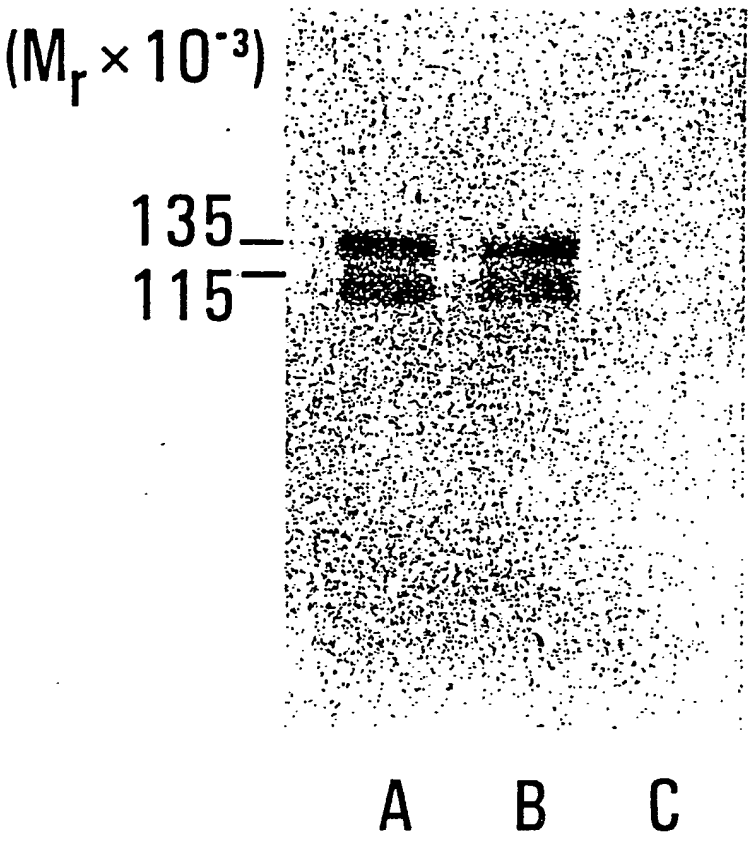

FIGURE 4

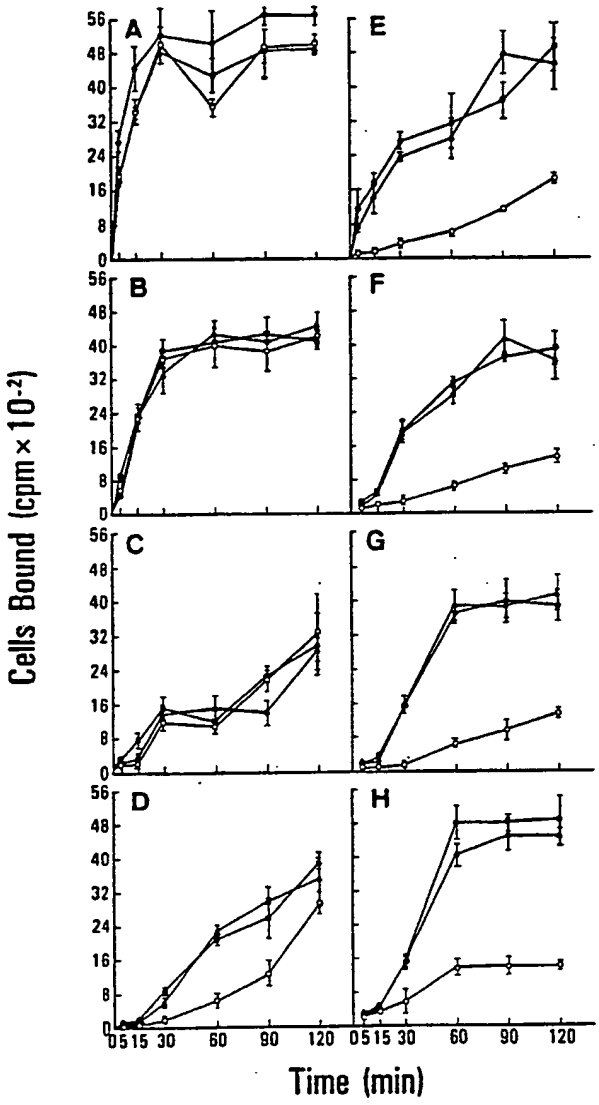
A
B
C
D
E
F
G
H
Cells Bound (cpm × 10⁻²)
0 8 16 24 32 40 48 56
0 5 15 30 60 90 120
Time (min)

FIGURE 5

Amino Acid Sequence of the Amino Terminus
of RGD-Directed Adhesion Receptors

	5				10				15				20							
ALPHA SUBUNIT																				
						D	G								V					
ECr	F	N	L	D	V	E	S	P	A	E	Y	S	G	P	E	G	S	Y	F	
VNR	F	N	L	D	V	X	S	P	A	E	Y	S								
GpIIb	L	N	L	D	P	V	Q	L	T	F	Y	A	G	P	N	G	S	Q	F	G F S
BETA SUBUNIT																				
						S										G				
ECr	G	P	N	I	C	T	T	R	G	V	S	S	C	Q	Q	C	L			
VNR	G	P	N	I	C	T	T	S	G	V	S	S	C	Q						
GpIIIa	G	P	N	I	C	T	T	R	G	V	S	S	C	Q						

FIGURE 6

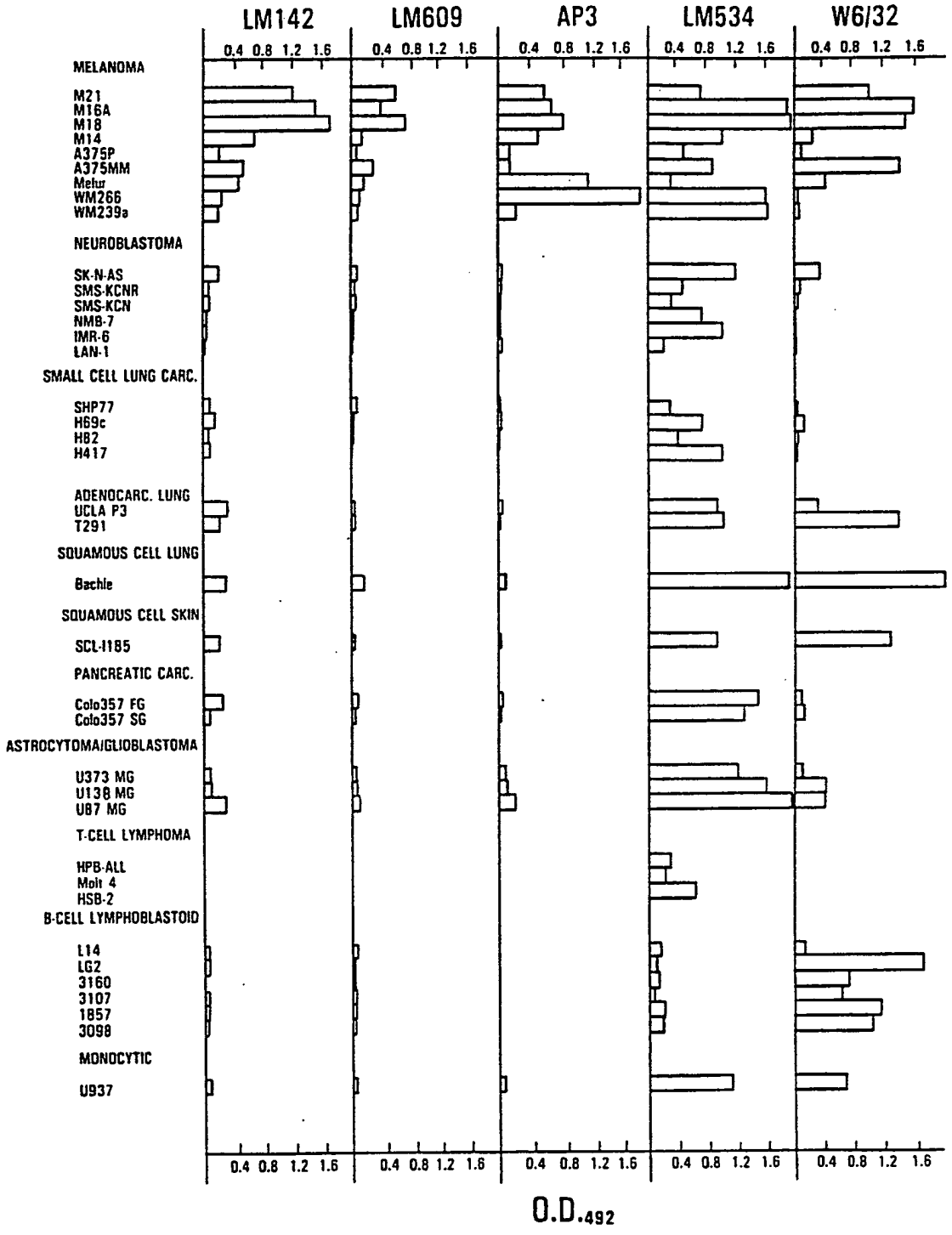
LM142
LM609
AP3
LM534
W6/32
0.4 0.8 1.2 1.6
MELANOMA
M21
M16A
M18
M14
A375P
A375MM
Metsu
WM266
WM239a
NEUROBLASTOMA
SK-N-AS
SMS-KCNR
SMS-KCN
NMB-7
IMR-6
LAN-1
SMALL CELL LUNG CARC.
SHP77
H69c
H82
H417
ADENOCARC. LUNG
UCLA P3
T291
SQUAMOUS CELL LUNG
Bachle
SQUAMOUS CELL SKIN
SCL-1185
PANCREATIC CARC.
Colo357 FG
Colo357 SG
ASTROCYTOMA/GLIOBLASTOMA
U373 MG
U138 MG
U87 MG
T-CELL LYMPHOMA
HPB-ALL
Molt 4
HSB-2
B-CELL LYMPHOBLASTOID
L14
LG2
3160
3107
1857
3098
MONOCYTIC
U937
O.D.492

FIGURE 7

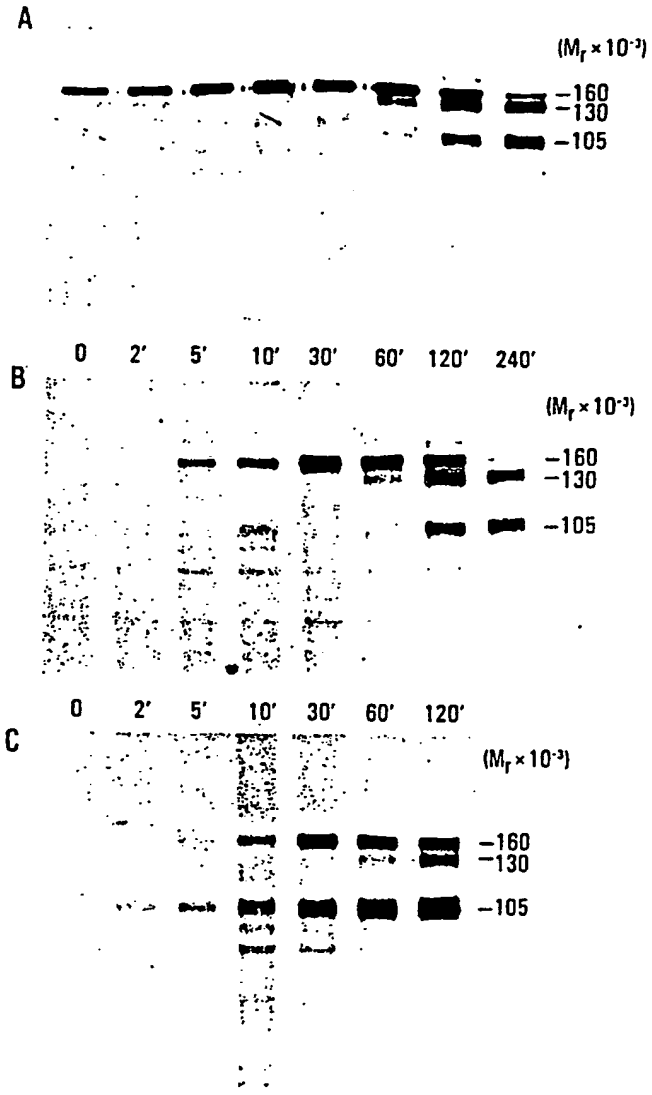
A
($M_r \times 10^{-3}$)
160
130
105
B
0 2' 5' 10' 30' 60' 120' 240'
($M_r \times 10^{-3}$)
160
130
105
C
0 2' 5' 10' 30' 60' 120'
($M_r \times 10^{-3}$)
160
130
105

FIGURE 8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/04118

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A61K 39/395, G01N 33/577, C12N 5/00, A61K 49/00

U.S. Cl.: 530/387; 435/240.27, 7; 424/9, 85.8

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/7, 172.2, 240.27 424/9, 85.8, 85.91 436/501, 512, 518, 548, 811 935/103, 107, 110 530/387, 808, 809

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Computer Search: CAS 1967-1989, Biosis 1977-1989

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	The Journal of Biological Chemistry, Volume 262, No. 21, issued 25 July 1987 (Baltimore, Maryland, USA), I. F. Charo, "Platelet Glycoprotein II-b-IIIa-like Proteins Mediate Endothelial Cell Attachment to Adhesive Proteins And The Extracellular Matrix," see page 9936, column 2, lines 15-25, page 9937, column 1, line 5-column 2, line 7 and page 9938, column 1, lines 10-24.	1-11
Y	The Journal of Biological Chemistry, Volume 262, No. 4, issued 05 February 1987 (Baltimore, Maryland, USA), D. A. Cheresh, "Arg-Gly-Asp Recognition By A Cell Adhesion Receptor Requires Its 130 KDa Subunit," see page 1434, column 2, lines 7-15, page 1435, column 1, lines 13-18, page 1437, column 1, lines 10-15 and line 41-column 2, line 7.	1-11

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 February 1989	26 APR 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Esther M. Kepplinger

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X,P Y,P	The Journal of Biological Chemistry, Volume 262, No. 36, issued 25 December 1987 (Baltimore, Maryland, USA), D. A. Cheresh, "Biosynthetic and Functional Properties of an Arg-Gly-Asp-directed Receptor Involved In Human Melanoma Cell Attachment To Vitronectin, Fibrinogen, and von Willebrand Factor", see page 17703, column 2, lines 1-5 and 24-43 and page 17710, column 1, lines 21-24 and column 2, lines 1-15.	1-3 4-11
Y	The Journal of Immunology, Volume 138, No. 10, issued 15 May 1987 (Baltimore, Maryland, USA), J.R. Ortaldo, "Analysis of Effector Cells In Human Antibody-Dependent Cellular Cytotoxicity With Murine Monoclonal Antibodies," see page 3566, column 2, lines 25-34, page 3567, column 2, lines 38-45 and page 3571, column 2, lines 31-43.	1-11